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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
Rab proteins are small GTPases that are essential elements of the protein transport machinery of eukaryotic cells. Each round of membrane transport requires a cycle of Rab protein nucleotide binding and hydrolysis. My research project consists in the study of Rab GTPases, the way in which they regulate intracellular transport, and the elucidation of mechanisms by which proteins involved in intracellular protein trafficking are linked to uncontrolled cellular proliferation and cancer. Our laboratory has recently characterized a protein, Yip1p, which appears to play a role in Rab-mediated membrane transport in *Saccharomyes cerevisiae*. In a search for a protein accesory factor that may act in conjunction with Yip1p, we discovered the Familial Adenomatous Polyposis (FAP) locus gene TB2. This gene is adjacent to tumor suppressor genes MCC and APC In this past year I have been able to characterize Yop1p the *Saccharomyces cerevisiae* homolog of TB2. Yop1p overexpression results in cell death and defects in intracellular trafficking. My project will be to characterize TB2 and its interaction with YIP1 and Rab proteins. Our studies will aim to dissect the molecular details of the TB2 gene and its interaction with other proteins through mutagenesis and molecular gene replacement. We will then characterize its role in the breast cancer cell line MCF7 and examine the physiological role of TB2 during mammary epithelial cell differentiation.

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(JBC Publication: Calero,M.,Whittaker,G.W., and Collins,R.N..(2001) *JBC* 276:12100-12112)

INTRODUCTION:

Rab proteins are small GTPases that are essential elements of the protein transport machinery of eukaryotic cells. Each round of membrane transport requires a cycle of Rab protein nucleotide binding and hydrolysis (1). My research project aims to examine the mechanisms by which Rab GTPases regulate intracellular transport, and the elucidation of the links connecting intracellular protein traffic to uncontrolled cellular proliferation and cancer. Our laboratory has recently characterized a protein, Yip1p, which appears to play a role in Rab-mediated membrane transport in *Saccharomyes cerevisiae* (unpublished results). In a search for a protein accessory factor that may act in conjunction with Yip1p, we discovered the Familial Adenomatous Polyposis (FAP) locus gene TB2. FAP is one of the most common autosomal dominant diseases leading to cancer predisposition affecting 1 in 5,000 individuals in the USA (2). A region on chromosome 5q21 is linked to both the inheritance of FAP and to tumor development in patients with non-inherited forms of cancer. These genetic lesions are some of the earliest events identified in sporadic cancers and are generally thought to indicate the presence of a tumor suppressor gene in the deleted region. It is not known whether TB2 is directly linked to tumorigenesis, as the majority of studies have examined the roles of the two adjacent genes MCC and APC which are also located in this region and are found to be altered in cancer patients with both inherited and sporadic forms of the disease (3).

I am focusing my attention to the characterization of the TB2 and its interaction with Yip and Rab proteins. Disruption of *YOP1* (the *S.cerevisiae* homolog) has no apparent effect on cell viability, while over-expression results in cell death and accumulation of internal cell membranes.

BODY:

This first year of my research has been very productive. A characterization of TB2 homolog in *S. cerevisiae*, *YOP1* resulted in a publication in the Journal of Biological Chemistry (4). Briefly, in this manuscript we have shown that Yop1p is a 20kDa integral membrane protein. Furthermore, we show that overexpression of *YOP1* is dominant negative for growth and results in accumulation of internal cell membranes, alteration of membrane structures and a block in membrane traffic. We also show a complete genetic and biochemical analysis of the Yop1p/Yip1p interaction and concluded that these two proteins interact in vivo, in a complex that is formed prior to cell lysis. In addition we show cellular localization of *Yop1p* in yeast by immunofluorescence.

An objective in my statement of work was to focus our studies on TB2 and potential interactions with Rab proteins in particular Rab3A. We were particularly interested in Rab3A because Vadlamudi and collegues reported that heregulin B1 (HRG), a combinatorial ligand for human growth factor receptors 3 and 4 that promotes the differentiation of mammary epithelial cells into secretory lobuloalveoli, promotes Rab3A expression and the accumulation of Rab3A-associated vesicles in MCF7 breast cancers cells (5). We decided to take a more global approach at first and investigate the interaction of yTB2 (Yop1p) with a comprehensive group of Rab proteins. There are over 30 Rab proteins in mammalian cell and 11 Rabs in yeast which are named Ypts (Yeast protein transport) (5). Nine of the yeast Rabs have a defined role in different steps of the yeast protein transport pathway and have been shown to share 54-71% identity with their closest mammalian Rab homologues (6). This conservation also extends to their function as well, as Ypt proteins and their Rab homologs act in similar intracellular compartments and, frequently, in similar transport steps (7). To get an overall idea of the kind of interactions that TB2 might have with Rab GTPases, we made GST fusions with several Rab proteins. The Rab GST-fusions include representatives from each subset of the Rab family. These constructs were expressed in cells behind the galactose promoter and were tested for interaction by coprecipitation with Yop1p. The results from these experiments are depicted in figure 10 of the manuscript (4). We clearly demonstrate that Yop1p can interact with all of the Rab proteins tested. No interaction was observed with a Ypt1p construct lacking its C-terminal cysteines which are the sites of C-terminal prenylation. Now we have a better understanding of the kind of the interaction between Yop1p and Rab proteins, we will now begin our studies in mammalian cells.

Another goal in my statement of work was a screen for other genes that could suppress the growth phenotype of *YOP1* overexpression. We recently obtained a 2μ library containing the entire yeast genome to begin to do the suppression analysis. However, we began this analysis by cloning all of the 11 yeast Rabs into multi-copy 2μ plasmids. The results of these experiments as shown in the manuscript, figure 9. While *YIP1* was able to suppress the dominant negative phenotype, none of the Rabs suppressed the full length *YOP1* dominant negative construct. However, multi-copy *YPT6* was able to suppress the dominant negative phenotype of the *YOP1* C-terminal construct. In the manuscript we explain that the C-terminus of the

molecule is mainly hydrophobic and corresponds to the second genomic exon. Furthermore, its overexpression also leads to inhibition of cell growth.

Another objective in my statement of work was to continue the analysis of *YOP1* with mutagenesis and phenotypic analysis. We report electron microscopy on cells overexpressing *YOP1* full length . (Figure 4 and 6 in the paper). We show that cells overexpressing *YOP1* resulted in the disappearance of large vacuoles normally seen in wild type cells and the appearance of smaller aberrantly shaped compartments filled with darkly stained material. Such aberrant structures are not present in wild type cells and represent a gross distortion of the normal pathways of membrane traffic in the *YOP1* dominant negative cells. Furthermore, we provide evidence on defects in exocytosis by monitoring the steady state level of newly synthesized precursors of the vacuolar protease CPY. These results are shown in Figure 7 of the manuscript. Cells overexpressing Yop1p full length accumulated a core-glycosylated ER form which is indicative of a block early in exocytosis We also show light microscopy (DIC images) on cells overexpressing *YOP* full length and *YOP1* C terminus. Cells overexpressing Yop1p full length are huge and swollen while *YOP1* C terminus overexpression leads to tiny cells. We will now begin mutagenesis analysis to further investigate the phenotypes of *YOP1*.

A last objective stated in my statement of work was to begin to prepare antibodies against TB2 and Yop1p. We have cloned these genes fused to HA and GST into expression vectors and will begin the purification to raise antibodies against them.

KEY RESEARCH ACCOMPLISHMENTS:

- Investigation of the phenotypes and morphological alterations of cells overexpressing YOP1
- Physical characterization of Yop1p.
- Biochemical and genetic analysis of Yop1p/Yip1p interactions
- Analysis of Yop1/Rab interactions
- Localization of Yop1p in yeast.
- Cloning of Yop1p and TB2 into expression vectors to begin antibody preparation.

REPORTABLE OUTCOMES:

Outcomes that have resulted from this research :

1. Journal article publication : Calero,M.,Whittaker,G.W., and Collins,R.N..(2001) "Yop1p, the Yeast Homolog of the Polyposis Locus Protein 1, Interacts with Yip1p and Negatively Regulates Cell Growth". *JBC* **276**:12100-12112.

2. Abstract: Calero,M,Whittaker,G.W. and Collins,R.N "Yop1p: A Novel Memebrane Protein in Yeast which negatively regulates cell growth and binds the Rab-interacting protein Yip1p" *MCB* Abstracts for ASCB meeting. Poster presented at the 40th Annual Meeting of The American Society for Cell Biology (ASCB): "Yop1p: A Novel Memebrane Protein in Yeast which negatively regulates cell growth and binds the Rab-interacting protein Yip1p"

3. Poster presented at the annual meeting of the field of Pharmacology, Cornell University: "Yop1p: A Novel Memebrane Protein in Yeast which negatively regulates cell growth and binds the Rab-interacting protein Yip1p"

CONCLUSIONS:

There is a growing appreciation that many proteins involved in intracellular protein trafficking are linked to uncontrolled cellular proliferation by diverse mechanisms (9-11). It is not known whether TB2 is directly linked to tumorigenesis, as the majority of studies have examined the roles of the two adjacent genes MCC and APC (12). The role of TB2 therefore remains an open question. Our genetic data suggest that *YOP1* has the properties expected of a tumor suppressor gene, which negatively regulates cell growth. Deletion of *YOP1* has no apparent effect on cell viability and overexpression of the gene results in cell death. Tumor suppressor genes can be differentiated from other classes of recessive genes with negative growth effects such as malignancy suppressor genes or cellular senescence genes by their possession of a dose-dependent growth inhibitory effect. These data indicate that the normal mode of action of Yop1p is as a recessive negative regulator, which is the role performed by a tumor suppressor protein in metazoan organisms. Strikingly, overexpression of the Yop1p C-terminus alone lacking the Yip1p interaction region leads to inhibition of growth with a phenotype of small cell size. This is reminiscent of transgenic expression of wildtype growth inhibitory tumor suppressor genes such as *RB*, *p53* and *p21^{CIP1}* which also result in cells of smaller size (12-15).

The ultimate goal of my research is to elucidate the role of TB2 and its relationship with Rab GTPases. Our data indicate that *YOP1* and possibly TB2 may play a role in the regulation of cell growth through its facilitation of membrane traffic. This first year I have been able to make a lot of progress in characterizing the yeast homolog of TB2. My next plan is to perform mutagenesis studies on YOP1 and to move into the mammalian system to look at mammalian Rabs, yeast homologs of Yip1p and their relationship to TB2. MCF7 cell will be used as a model and particular interest will be paid to Rab3A.

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Yop1p, the Yeast Homolog of the Polyposis Locus Protein 1, Interacts with Yip1p and Negatively Regulates Cell Growth*

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Rab proteins are small GTPases that are essential elements of the protein transport machinery of eukaryotic cells. Each round of membrane transport requires a cycle of Rab protein nucleotide binding and hydrolysis. We have recently characterized a protein, Yip1p, which appears to play a role in Rab-mediated membrane transport in *Saccharomyces cerevisiae*. In this study, we report the identification of a Yip1p-associated protein, Yop1p. Yop1p is a membrane protein with a hydrophilic region at its N terminus through which it interacts specifically with the cytosolic domain of Yip1p. Yop1p could also be coprecipitated with Rab proteins from total cellular lysates. The *TB2* gene is the human homolog of Yop1p (Kinzler, K. W., Nilbert, M. C., Su, L.-K., Vogelstein, B., Bryan, T. M., Levey, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKechnie, D., Finniear, R., Markham, A., Groffen, J., Boguski, M. S., Altschul, S. F., Horii, A., Ando, H. M., Y., Miki, Y., Nishisho, I., and Nakamura, Y. (1991) *Science* 253, 661–665). Our data demonstrate that Yop1p negatively regulates cell growth. Disruption of *YOP1* has no apparent effect on cell viability, while overexpression results in cell death, accumulation of internal cell membranes, and a block in membrane traffic. These results suggest that Yop1p acts in conjunction with Yip1p to mediate a common step in membrane traffic.

The Rab family encompasses a conserved group of key molecules involved in membrane traffic and represents a distinct subgroup of the Ras superfamily (2). Each stage of membrane traffic through both the constitutive and regulated secretory pathways of all eukaryotic cells is associated with a distinct Rab protein that regulates the cascade of events that lead to SNARE-mediated membrane fusion (3). A hallmark of Rabs is their localization to specific compartments of the transport pathway. This distribution is consistent with the function of Rab proteins in distinct intracellular transport processes. In every case examined, the localization pattern of a Rab protein reflects the membrane transport step that it regulates. In keeping with this view, more than 30 members of the Rab family have been identified (2).

Rabs are stably prenylated at their C terminus, which mediates their association with membranes (4). However, while the majority of Rabs are membrane-associated, prenylated Rabs are also found in the cytosol bound to the Rab GDP dissociation inhibitor (GDI).[1] GDI shares sequence homology with the Rab escort protein involved in presenting and removing Rab proteins from the prenylation machinery (5, 6). GDI has several properties that underscore its role in mediating Rab protein function: (i) GDI binds preferentially to the GDP-bound conformation of Rab proteins and slows the intrinsic rate of GDP nucleotide dissociation (7), (ii) GDI requires the fully prenylated Rab protein for interaction and binds in such a way so that the geranylgeranyl groups are shielded in a hydrophobic pocket (8), (iii) GDI is a pleiotropic factor interacting with many different Rab proteins *in vitro* and *in vivo*; in *Saccharomyces cerevisiae*, a single gene encodes GDI function for all 11 Rab proteins (9). These properties enable the Rab protein to exist in the aqueous environment of the cytoplasm as a soluble heterodimer with GDI and facilitate recycling of the GDP-bound Rab back to the donor compartment (10). Consistent with this model, Rab proteins are complexed to GDI in the cytosol, and depletion of GDI in yeast causes loss of the soluble pool of Rabs and a concomitant inhibition of transport in the secretory pathway.

The specificity of Rab protein function, localization, and their presence on the surface of vesicles suggests the existence of a machinery that recruits Rab proteins to the proper target membrane. However, identification of such a machinery has proven elusive. To date, no factor mediating this process has been identified; however, several features of Rab membrane recruitment have been established: (i) Rabs are recruited to membranes in their inactive GDP-bound conformation bound to GDI (11); (ii) membrane recruitment is accompanied by the displacement of GDI (12); (iii) membrane recruitment is specific, and the C-terminal hypervariable region of the Rab protein mediates this specificity (13); (iv) prenylation of Rab proteins is crucial for membrane recruitment in addition to the C-terminal ~35 amino acid residues; (v) membrane recruitment is followed by nucleotide exchange, and the two processes can be distinguished kinetically (14, 15); and (vi) for Rab4, the existence of a membrane protein that acts as a specific Rab receptor has been demonstrated, although the precise identity of this receptor is unknown (16).

We have characterized a membrane protein in yeast, Yip1p, which appears to mediate the dissociation of the Rab het-

[1] The abbreviations used are: GDI, GDP dissociation inhibitor; GST, glutathione *S*-transferase; HA, influenza virus hemagglutinin epitope; PCR, polymerase chain reaction; ORF, open reading frame; PBS, phosphate-buffered saline; PNS, postnuclear supernatant; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; CPY, carboxypeptidase Y.

TABLE I
S. cerevisiae strains used in this study

Strain	Genotype	Source
NY605	*MATa ura3–52 leu2–3,112*	Novick laboratory
RCY376	*MATa ura3–52 leu2–3,112::LEU2 GAL$_{1-10}$→HA-YOP1*	This study
RCY377	*MATa ura3–52 leu2–3,112::LEU2 GAL$_{1-10}$→HA-YOP1 C terminus*	This study
RCY404	*MATa leu2–3,112 ura3–52 YOP1::URA3 GFP-YOP1*	This study
RCY407	*MATa ura3–52 leu2–3,112 YOP1ΔKAN^R*	This study
RCY423	*MATa ura3–52 leu2–3,112::LEU2 GAL$_{1-10}$→GST-YOP*	This study
RCY425	*MATa ura3–52 leu2–3,112::LEU2 GAL$_{1-10}$→GST-YOP1 [URA3 2μ myc$_9$-YIP1]*	This study
RCY427	*MATa ura3–52 leu2–3,112::LEU2 GAL$_{1-10}$→GST*	This study
RCY428	*MATa ura3–52 leu2–3,112::LEU2 GAL$_{1-10}$→GST [URA3 2μ myc$_9$-YIP1]*	This study
RCY429	*MATa ura3–52 leu2–3,112::LEU2 GAL$_{1-10}$→GST-YOP1 [URA3 2μ DSS4-myc$_3$]*	This study
RCY460	*MATa ura3–52 leu2–3,112 YOP1ΔKAN^R [URA3 CEN HA-YOP1]*	This study
RCY462	*MATa ura3–52 leu2–3,112 [URA3 2μ myc$_9$-YIP1]*	This study
RCY469	*MATa leu2–3,112 ura3–52::URA3 HA-YOP1 YOP1ΔKAN^R*	This study
RCY496	*MATa leu2–3,112 ura3–52::URA3 HA-YOP1 [LEU2 CEN GFP-YIP1] YOP1ΔKAN^R*	This study
RCY509	*MATa leu2–3,112 ura3–52 [URA3 2μ GAL$_{1-10}$→GST-YOP1] [LEU2 CEN GFP-YIP1]*	This study
RCY508	*MATa leu2–3,112 ura3–52 [URA3 2μ GAL$_{1-10}$→GST-YOP1] [LEU2 CEN GFP-YPT7]*	This study
RCY455	*MATa/α ura3–52 his3Δ200 leu2–3,112::LEU2 GAL$_{1-10}$→GST-YPT52 [pRS426 GAL$_{1-10}$→HA-YOP1 (pRC782)]*	This study
RCY456	*MATa/α ura3–52 his3Δ200 leu2–3,112::LEU2 GAL$_{1-10}$→GST-SEC4 [pRS426 GAL$_{1-10}$→HA-YOP1 (pRC782)]*	This study
RCY457	*MATa/α ura3–52 his3Δ200 leu2–3,112::LEU2 GAL$_{1-10}$→GST-YPT1ΔC [pRS426 GAL$_{1-10}$→HA-YOP1 (pRC782)]*	This study
RCY465	*MATa/α ura3–52 his3Δ200 leu2–3,112::LEU2 GAL$_{1-10}$→GST-YPT6 [pRS316 GAL$_{1-10}$→HA-YOP1 (pRC783)]*	This study
RCY467	*MATa/α ura3–52 his3Δ200 leu2–3,112::LEU2 GAL$_{1-10}$→GST-YPT7 [pRS316 GAL$_{1-10}$→HA-YOP1 (pRC783)]*	This study
RCY464	*MATa/α ura3–52 his3Δ200 leu2–3,112::LEU2 GAL$_{1-10}$→GST [pRS316 GAL$_{1-10}$→HA-YOP1 (pRC783)]*	This study
Y190	*MATa gal4Δgal80Δ trp1–901 ade2–101 ura3–52 leu2–3,112 URA3::GAL10→LacZ, LYS2::GAL10→HIS3 cyh^R*	Elledge laboratory

erodimer from GDI. *YIP1* is an essential gene (17) that is highly conserved in evolution.[2] However, Yip1p is a pleiotropic factor and lacks specificity for interaction with any particular Rab GTPase (17). We have therefore searched for a protein accessory factor that may act in conjunction with Yip1p, and we report the identification of a novel membrane protein, Yop1p, which physically interacts with Yip1p. Disruption of *YOP1* has no apparent effect on cell viability, while overexpression results in cell death and accumulation of internal cell membranes. These results suggest that Yop1p acts in conjunction with Yip1p to mediate a common step in membrane traffic. Because of the essential nature of Rab recruitment for the activation and recycling of Rabs, characterization of Yop1p may provide crucial insight into the action of Rab proteins in mediating membrane transport.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The *S. cerevisiae* strains used in these studies are listed in Table I. All yeast strains were manipulated as described by Guthrie and Fink (18). *YOP1* gene deletion was carried out using the *KAN^R* module (19) as a selectable marker and the primers CAAAGACATAACCGCACTCCAATCATGTCCGAATATGCATCTAGT-ATTCACTCTCCGTACGCTGCAGGTCGAC and GAGGATATAGGTG-AGTTGCCTCTTAATGAACAGAAGCACCTGTAGCCTTAGAAGCCTA-TCGATGAATTCGAGCTCG to precisely eliminate the *YOP1* ORF. Genomic PCR using an internal deletion primer and the flanking primer CTTGAAGCTTGTTATTCCGA was performed to verify gene disruption. Yeast expressing GST-Yop1p under the control of the GAL$_{1/10}$ promoter (RCY423) and GST alone (RCY427) were created by digesting pRC494 and pRC337, respectively, with *ClaI* to direct integration at the *LEU2* locus of NY605. Strains RCY425, RCY428, and RCY462 were created by transforming pRC695 into RCY423, RCY427, and NY605, respectively. In the same manner, RCY429 was created by transforming pNB632 into RCY423. RCY460 was created by transforming the hemagglutinin (HA)-tagged Yop1p protein expression vector pRC778 into RCY407. For immunofluorescence, RCY469 was created by digesting pRC833 with *EcoRV* to direct integration of the plasmid at the *URA3* locus of RCY407.

Yeast were streaked out on a selective plate and incubated at

30 °C. Liquid media cultures were grown at room temperature. A single colony from each strain was inoculated into 5 ml of selective medium and grown to stationary phase. The day prior to the experiment, medium was inoculated with aliquots of stationary culture at room temperature to obtain cells in logarithmic phase growth. Turbidity measurements were made using a Beckman model DU-40 spectrophotometer at 600 nm.

Plasmids and DNA Constructs—The genomic *YOP1* ORF contains a single intron. For convenience, this intron was removed for the majority of *YOP1* constructs by overlap PCR with the primers RNC66 (GGAG-CTCCACCGCGGTGGCGGCCGCTCTAGAACTAGGAAGAGTTGCAT-AGATAGGATGGGTGA) and RNC78 (CGATACCAAGTACTCTGGTA-ATAGAATTTTACAGC) together with RNC67 (CTCGAGGTCGACGG-TATCGATAAGCTTGATATCGAATGCTCAAAAGCTAACACTAGGCC-AG) and RNC79 (TATCCATGGGTAAGTACTCTGGTAATAGAATTTT-ACAGC). Full-length *YOP1* fusion constructs were constructed by PCR with oligonucleotides RNC44 (TGGTACCTCATGAGCGAATATGCAT-CTAGTATTCACTCTC) and RNC80 (AATAGGATCCTTAATGAACAG-AAGCACCTGTAG). The *NcoI/BamHI*-digested PCR product was subcloned into pAS2-1 and pACT2 to create two-hybrid vectors expressing full-length *YOP1*, p121 to create HA-tagged *YOP1* under the control of the GAL$_{1/10}$ promoter (pRC393) and pRC337 to create GST-tagged *YOP1* under the control of the GAL$_{1/10}$ promoter (pRC494). C-terminal *YOP1* constructs containing amino acids 18–180 were created in a similar manner with the oligonucleotides RNC79 (TATCCATGGGTA-AGTACTCTGGTAATAGAATTTTACAGC) and RNC80. The PCR product was subcloned into p121 to create pRC439 expressing HA-tagged *YOP1* C terminus under the control of the GAL$_{1/10}$ promoter. pRC581 containing yEGFP-tagged *YOP1* under the control of its own gene regulatory elements in pRS406 was created by overlap PCR with the oligonucleotides RNC66, RNC67, RNC179 (CAAAGACATAACCGCAC-TCCAATCATGTCTAAAGGTGAAGAATTATTC), RNC180 (AGAGTG-AATACTAGATGCATATTCGGATTTGTACAATTCATCCATACC), RNC181 (CATGATTGGAGTGCGGTTATG), and RNC182 (TCCGAAT-ATGCATCTAGTATTCACTCTCAAATGAAAC). pRC695 expressing Myc-*YIP1* in pRS426 (20) was created by overlap PCR placing a cassette containing Myc$_9$ (gift of Y. Barral, ETH, Zurich) in frame behind the start codon of *YIP1* with the primers 9× oligo 1 YIP1 (GCAAGAC-AACTATTAGTCCCTCTCGAGATGCTCCACCGCGGTGGC) and 9× oligo 2 YIP1 (TGTTACTAGTATTGTAGAAAGACATAATTCCTGCAG-CCCGGGGGAT). pNB632, a *URA3* multicopy plasmid containing *DSS4*-Myc$_3$, has been described previously (21). pRC337 was created by subcloning GST in front of the GAL$_{1/10}$ promoter of vector pNB527 digested with *BamHI/XhoI* using primers RNC177 (CTAGACTAGAT-

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[2] R. Collins, unpublished data.

CTTCATGAGTTCCCCTATACTAGGTTATTGGAAAATTAAG) and RNC178 (GACTGACCTCGAGTAGGATCCAGTCACCATGGTCAGAT-CCGATTTTGGAGGATG) and digesting the PCR product with *Bgl*II/*Xho*I. pRC778 containing a single HA epitope at the N terminus of Yop1p expressed at wild-type levels in the vector pRS315 was created by PCR overlap with the oligonucleotides RNC157 (TACGACGTCCC-AGACTACGCTTCCGAATATGCATCTAGTATTCAC) and HA 1 (AGC-GTAGTCTGGGACGTCGTATGGGTACATCTCGAGAGGGACTAATA-GTTGTC). The insert of pRC778 was removed with *Sal*I/*Hin*dIII and ligated into the vector pRS306 digested with *Xho*I/*Hin*dIII to create pRC833, a *URA3*-integrating vector expressing wild-type levels of HA-tagged Yop1p. pRC693 containing green fluorescent protein (GFP)-tagged Yip1p under the control of its own promoter and terminator in pRS315 was constructed by placing a cassette containing yeast-enhanced GFP mut3 (22) in frame behind the start codon of *YIP1* with the primers GFP oligo 1 (GACAACTATTAGTCCCTCTCGAGATGTCTAA-AGGTGAAGAATTATTCAC) and GFP oligo 2 (GTTACTAGTATTGTA-GAAAGACATTTTGTACAATTCATCCATACCAT). pRC650 and pRC556 containing GFP-tagged Ypt6p and Sec4p, respectively, in pRS315 were created in a similar fashion. pRC903 was created by subcloning a cassette containing GST-tagged *YOP1* under the control of the $GAL_{1/10}$ promoter from pRC494 into the 2μ *URA3* vector pRS426. pRC940 containing *YIP1* was constructed by genomic PCR with the oligonucleotides YF YIP1 (GT-ACCGGGCCCCCCCTCGAGGTCGACGTAGTGCTTGTTACGTTAG) and YR YIP1 (CCACCGCGGTGGCGGCCGCTCTAGAACTCTATGCT-TTCCTTATTTACCTCTGGA) and inserted into pRS426 to create a multicopy *URA3* vector.

Electrophoresis and Western Blotting—For electrophoresis, samples were boiled for 5 min in gel loading buffer (60 mM Tris, pH 6.8, 10% sucrose, 2% SDS, 5% β-mercaptoethanol, and 0.005% bromphenol blue), microcentrifuged for 5 min, and loaded onto 12 or 14% SDS-polyacrylamide gels (37.5:1 acrylamide/bisacrylamide). Prestained protein molecular weight markers were from Life Technologies, Inc. For Western blotting, gels were transferred to polyvinylidene difluoride membranes for 2 h at 200 mA. The membranes were stained with Ponceau S to observe the quality of the transfer. Antigens on the membrane were detected by incubating the filter with blocking buffer (5% nonfat dry milk in TBST; 150 mM NaCl, 50 mM Tris, pH 7.5, and 0.2% Tween 20). Primary antibodies were incubated in TBST, followed by three washes. Secondary alkaline phosphatase-conjugated antibodies were added in blocking buffer, followed by three washes and chromogenic blot development with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (both from Bio-Rad) substrates in AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$).

Coprecipitation Assays—Yeast strains were grown in minimal medium containing 2% galactose. 10 OD units from each culture were harvested and washed in 1 ml of ice-cold TAZ buffer (10 mM Tris, pH 7.5, 10 mM NaN_3). Cell pellets were then resuspended in 100 μl of ice-cold lysis buffer (20 mM KPi, 80 mM KCl, 1 mM EDTA, 2% glycerol, 0.1% Tween 20) containing protease inhibitors (10 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A), and an equal volume of glass beads was added. The cells were then lysed by vortexing for 2 min in a Turbo-Beater (Fisher) at 4 °C. A total detergent-solubilized lysate was generated by incubating lysates end-over-end with an additional 1 ml of lysis buffer for 10 min at 4 °C. Detergent-solubilized lysates were cleared by two sequential centrifugation steps in a microcentrifuge for 5 min at 13,000 rpm. 20 μl of glutathione *S*-transferase 4B beads (GST-beads; Amersham Pharmacia Biotech) was added to the lysates and incubated with constant mixing for 30 min at 4 °C. After four washes with 0.6 ml of lysis buffer, the GST beads were boiled with SDS-PAGE sample buffer, and the samples were analyzed by SDS-PAGE and Western blot. Pull-down experiments from yeast strains RCY509 and RCY508 used the lysis buffer 25 mM KPi, pH 7.5, 160 mM KCl, 2 mM EDTA, 2% glycerol, and 0.4% Triton X-100. Pull-down experiments fom yeast strains RCY455, RCY456, RCY457, RCY465, RCY467, and RCY464 used the lysis buffer, 25 mM KPi, pH 7.5, 160 mM KCl, 2 mM EGTA, 2% glycerol, and 0.5% Tween 20. Primary antibodies used were rabbit polyclonal α-GST (gift of T. Fox, Cornell University), mouse monoclonal α-Myc antibody (9E10; Ref. 23), affinity-purified Rabbit α-GFP antibody (24) (gift of P. Silver, Dana-Farber Cancer Institute), and mouse monoclonal α-HA 12CA5. Alkaline phosphatase-conjugated anti-rabbit and anti-mouse secondary antibodies were used (Bio-Rad) to detect the presence of Myc_9-Yip1p and either GST alone or GST-Yop1p.

Subcellular Fractionation—Yeast strain RCY460 containing wild-type levels of HA-tagged Yop1p as the only source of *YOP1* was used for this experiment. 25 OD units were harvested and washed in 1 ml of TAZ buffer. Cells were broken by glass bead lysis in a Turbo-Beater at 4 °C

β-Galactosidase activity was determined by filter assay. Pairs were coexpressed in the reporter strain Y190. Plus represents a positive activity rated according to the following criteria: + + +, activity detected after 30 min; + +, activity detected after 90 min; and +, activity detected after 5 h. −, a negative indication of activity. At least 30 independent transformants were tested for each pair. Yop1p N-terminal constructs contain amino acids 1–17, and Yop1p C-terminal constructs contain amino acids 18–180. Yip1p N-terminal constructs contain amino acids 1–117. ND, not detected.

| Bait construct	Fish construct			
	Yip1p N-terminus	Yip1p full length	Yop1p N-terminus	Yop1p full length
Yip1p N terminus	−	−	+ + +	ND
Yip1p full length	−	−	+ + +	−
Yop1p N terminus	+ + +	+ + +	−	−
Yop1p C terminus	−	−	−	−

in fractionation buffer with protease inhibitors (PBS containing 0.2 M sorbitol and 1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin). A postnuclear supernatant (PNS) was generated by two sequential centrifugation steps for 5 min at 500 × g. 2.7 mg of PNS was then spun sequentially at 10,000 × g for 15 min and at 100,000 × g for 12 min to generate P10 and P100 fractions. For Triton X-100 solubilization, the P100 membrane pellet was resuspended in fractionation buffer containing 1% Triton X-100. Samples were incubated for 10 min on ice and recentrifuged at 100,000 × g. For high salt treatment, the P100 membrane pellet was resuspended in fractionation buffer containing 1 M NaCl. Samples were incubated for 10 min on ice and recentrifuged at 100,000 × g. Pellets and supernatants were resuspended in sample buffer and analyzed by SDS-PAGE and Western blot. The HA-Yop1p was detected with mouse monoclonal 12CA5 antibody followed by anti-mouse alkaline phosphatase-conjugated secondary antibody.

Triton X-114 Phase Separation—Triton X-114 (Roche Molecular Biochemicals) was purified by precondensation as described (25). 25 OD units of yeast strain RCY460 were harvested and washed in 1 ml of TAZ buffer. Postnuclear supernatants were generated as described above. 1.8 mg of PNS was added to the same volume of PBS containing 2% Triton X-114 with protease inhibitors (1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, and 10 μg/ml pepstatin A). The samples were incubated for 20 min at 4 °C to solubilize membrane proteins. The lysates were incubated for 3 min at 30 °C followed by low speed centrifugation (700 × g) to separate the detergent-enriched and the soluble phases. This cycle was repeated a further two times with the detergent-enriched and soluble phases individually. The detergent phase was washed twice with PBS containing 0.05% Triton X-114 and the soluble phase with 10% Triton X-114. Samples were analyzed by SDS-PAGE and Western blot. Sncl/2p, an integral membrane protein, was used as a positive control and was detected with anti-Sncl/2p antisera (gift of P. Brennwald, Cornell University).

Carboxypeptidase Y Analysis—Yeast strains RCY376 and RCY377 containing HA-YOP1 full-length and C-terminal constructs (respectively) behind the galactose promoter, were grown in sucrose minimal medium to early log phase, before washing and resuspending in galactose minimal medium. At the indicated intervals, aliquots of 5 OD units were harvested for production of lysates. For the *sec18* experiments, cells were grown at room temperature until log phase before shifting an aliquot to the restrictive temperature (37 °C) for 1 h. Lysates were then boiled with SDS-PAGE sample buffer for 5 min and analyzed by SDS-PAGE and Western blot. The membrane was probed with polyclonal anti-carboxypeptidase Y (CPY) (gift from P. Brennwald).

Immunofluorescence Experiments—Yeast strains RCY469 containing HA-Yop1p and RCY407 (isogenic untagged control) were grown to early log phase in YPD medium. 2× fixative (2× PBS, 4% glucose, 40 mM EGTA, 7.4% formaldehyde) was added to an equal volume of medium containing 3 OD units of cells and incubated for 20 min at room temperature. Cells were then collected by centrifugation, resuspended in 5 ml of 1× fixative, and incubated for a further 1 h. The cells were washed twice in 2 ml of spheroplasting buffer (100 mM KP$_i$, pH 7.5, 1.2 M sorbitol) and then incubated in spheroplasting buffer containing 0.2% 2-mercaptoethanol and 0.08 mg/ml of zymolyase for 30 min at 37 °C with gentle mixing. 20 μl of the cell suspension was placed on individual wells of a polylysine-coated printed microscope slides (Carlson Scientific, Inc.) for 10 min. The cells were then washed three times with

A
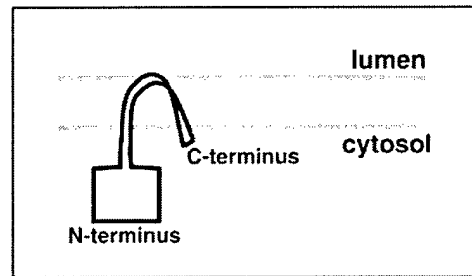
B
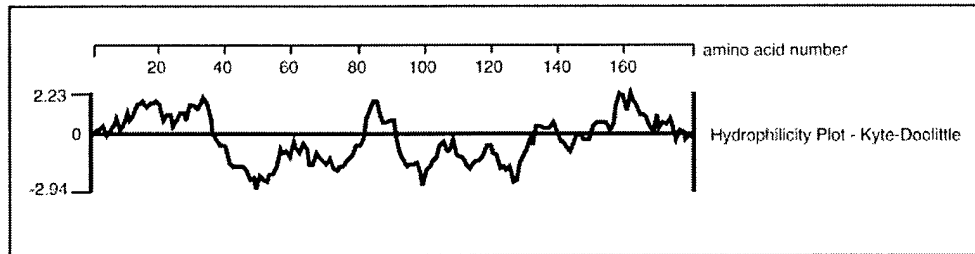
C

Fig. 1. *A*, schematic representation of Yop1p. Sequence data indicate a cytoplasmically oriented N terminus and a hydrophobic C-terminal domain that spans the membrane twice. B, Kyte-Doolittle hydrophobicity plot of Yop1p. This was generated using the program Protean (DNASTAR) with a 9-residue parameter average and shows the relative location of the two hydrophobic segments of the protein. *C*, alignment of Yop1p with data base homologs. Shown is the sequence of Yop1p and a comparison with human and murine TB2 and full-length cDNAs from other organisms. T41634 is from *Schizosaccharomyces pombe*, CG4960 and CG8331 are from *Drosophila melanogaster*, AAF36016 is from *Caenorhabditis elegans*, and CAN11144 is from *Plasmodium falciparum*. Mammalian expressed sequence tag fragments are not included in this alignment. The sequences were aligned using MegAlign. Amino acid residues are numbered according to the protein sequence. The *shaded residues* exactly match the consensus sequence.

PBS/BSA (1 mg/ml BSA) and permeabilized for 5 min with either 0.1% SDS or 0.1% Triton X-100 in PBS/BSA. After washing five times in PBS/BSA, cells were blocked for 30 min in PBS/BSA. Polyclonal α-HA antibody (Y11; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to each well at a dilution of 1:5000 and incubated for 1 h at room temperature. Cells were washed 5 times in PBS/BSA and then incubated with Texas Red-labeled anti-rabbit secondary antibody (Molecular Probes, Inc.) at a dilution of 1:200 for 30 min at room temperature. Monoclonal 1.2.3 antibody was used to detect Sec4p (26), and a monoclonal anti-GFP antibody (3E6; Molecular Probes, Inc., Eugene, OR)
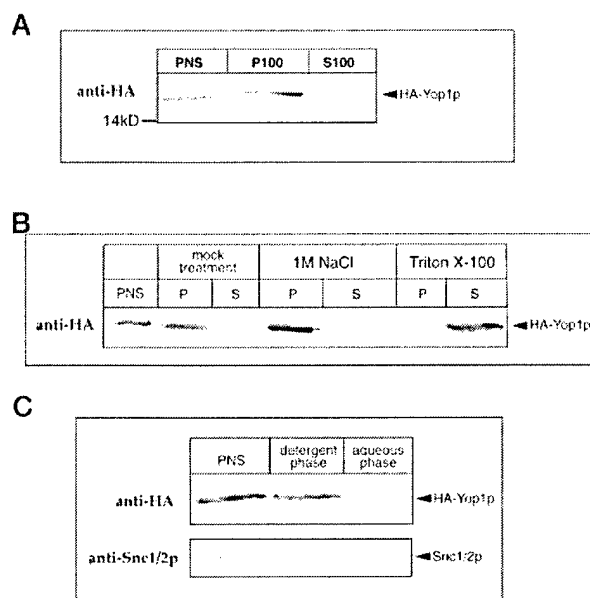
Identification of Yop1p

A

	PNS	P100	S100
anti-HA			◄HA-Yop1p
14kD—			

B

		mock treatment		1M NaCl		Triton X-100	
	PNS	P	S	P	S	P	S
anti-HA							◄HA-Yop1p

C

	PNS	detergent phase	aqueous phase
anti-HA			◄HA-Yop1p
anti-Snc1/2p			◄Snc1/2p

FIG. 2. *A*, membrane localization of Yop1p. Differential centrifugation was performed on logarithmically growing cells expressing HA-Yop1p at wild-type levels. Blots were probed for HA-Yop1p, which is present exclusively in the pellet fraction (P100; 100,000 × *g*). PNS represents a total postnuclear lysate (500 × *g* supernatant), and S100 is the supernatant remaining after the 100,000 × *g* centrifugation. *B*, high salt and Triton X-100 treatment of Yop1p-containing membranes. Postnuclear supernatants were centrifuged at 100,000 × *g* to obtain cytosolic and total membrane fractions. Total membrane fractions were resuspended in buffer with and without 1 M NaCl, 1% Triton X-100, or mock-treated before recentrifugation at 100,000 × *g*. The pellets and supernatants were dissolved in equivalent volumes of sample buffer and run on SDS-PAGE gel, and HA-Yop1p was detected by Western blotting. Relevant protein marker sizes are indicated. *C*, Triton X-114 phase separation of lysates expressing HA-tagged Yop1p. Triton X-114 fractionation generating a detergent-enriched phase and an aqueous phase was performed as described under "Experimental Procedures" on cells expressing HA-Yop1p at wild type levels. HA-Yop1p was detected by Western blotting and fractionates in the detergent-enriched phase. PNS represents total postnuclear supernatant. As a control, the fractions were probed for the membrane protein Snc1/2p, which is contained in the detergent-enriched phase. Relevant protein marker sizes are indicated on the *left*.

was used to detect GFP. These were followed by Oregon Green 514-labeled anti-mouse secondary antibody (Molecular Probes) at a dilution of 1:250. To stain nuclei, 5 μg/ml Hoechst 33258 (Molecular Probes) in PBS/BSA was added to each well, and after 10 min at room temperature, cells were washed five times. Cells were mounted in a small drop of mounting medium (Moviol), and the slides were left to air dry in the dark for at least 30 min. Confocal microscopy was performed using an Olympus FluoView confocal station (Olympus). Oregon Green was excited with the 488-nm line of an argon laser, and Texas Red was excited with the 568-nm line of a krypton laser.

Electron Microscopy—The cells were incubated for 14 h in medium containing galactose as sole carbon source at a final cell density (A_{600}) of between 0.4 and 0.7. Cells were washed with 0.1 M cacodylate, pH 6.8, and then fixed with 0.1 M cacodylate, pH 6.8, containing 3% glutaraldehyde for 1 h at room temperature and then overnight at 4 °C. The cell walls were removed by treatment with 0.1 M KP_i buffer, pH 7.5, containing 0.2 mg/ml zymolyase 100T. The cell pellet was incubated with 1.5 ml of cold 2% OsO_4 in 0.1 M cacodylate buffer for 1 h on ice followed by incubation with 1.5 ml of filtered 2% uranyl acetate (aqueous) at room temperature for 1 h. The cell pellets were dehydrated with the following ethanol washes: 50, 70, 90, and 100% followed by four washes from a fresh bottle of 200 proof ethanol and a final rinse in 100% acetone. The pellet was then incubated with 50% acetone, 50% SPURR resin (Electron Microscopy Sciences); this was changed to 100% SPURR resin, and the sample was transferred to beem capsules (Electron Microscopy Sciences) and baked at 80 °C for at least 24 h. Thin sections were cut onto Specimen Grids (Veco) (3-mm diameter, 75 × 300 mesh

copper), contrasted with lead citrate and uranyl acetate, and then examined in an FEI Philips TECHNAI 12 BioTwin electron microscope at 100 or 80 kV.

Two-hybrid Experiments—The ORF sequences were subcloned into pAS1-CYH2 or pAS2–1 for "bait" and pACTII for "fish" constructs, respectively. The yeast strain Y190 was used for to screen the library for N-terminal Yip1p-interacting clones (27). The yeast reporter strain Y190, which contains the reporter genes *lacZ* and *HIS3* downstream of the binding sequences for Gal4, was sequentially transformed with the pACT2 and pAS2–1 (CLONTECH) plasmids containing the genes of interest. Double transformants were plated on selective medium (lacking tryptophan and leucine) and incubated for 2–3 days at 30 °C. Trp+ Leu+ colonies processed for the β-galactosidase filter assay as described (21).

RESULTS

The Cytosolic Domain of Yip1p Interacts with a Novel Membrane Protein—To explore the role of Yip1p in membrane traffic, we considered the possibility that it may exist in physical association with other proteins. Such a protein may perhaps act to provide a specificity component to the Rab membrane recruitment reaction. To identify such potential proteins, we performed a two-hybrid screen using the cytosolic domain of Yip1p as bait. For this interaction screen, we used two-hybrid libraries constructed from short fragments (0.5–1 kilobase pair) of yeast genomic DNA (28). Since the yeast genome is relatively compact with few intron-containing genes, such a library represents a collection of random protein fragments. The rationale for such a strategy was that a Yip1p-interacting protein may be a membrane protein interacting with Yip1p through exposed soluble loops. Interactions may not be revealed by expressing full-length cDNAs, but protein fragments of the isolated loops alone may demonstrate interaction in the two-hybrid system. Analogous strategies have been used successfully to explore interactions of multispanning membrane proteins using the two-hybrid system (29). Using this screen, we identified a previously uncharacterized membrane protein derived from ORF *YPR028W*. The interacting clone identified contained 17 amino acids derived from the extreme N terminus of the protein fused in frame with the GAL4 DNA activation domain. We have termed this gene *YOP1* (YIP one partner). The interaction between the Yop1p fragment and Yip1p was recapitulated with a full-length Yip1p construct in the two-hybrid system. The interaction was also maintained whether or not the Yop1p fragment was a GAL4 DNA binding domain plasmid or a GAL4 DNA activation domain fusion; *i.e.* if the "bait" construct is swapped with the "fish" construct, the vast majority of false two-hybrid positives will not interact in such a test. However, Yop1p full-length constructs show no interactions with Yip1p in the two-hybrid system. These data are summarized in Table II.

The primary sequence of Yop1p is predicted to have at least two membrane-spanning domains (Fig. 1, *A* and *B*). A BLAST search of GenBank™ revealed that Yop1p is homologous to the human TB2 protein in addition to several other proteins present in data bases (Fig. 1C). Yop1p and human TB2 share 25.65% identity at the amino acid level, and there is 22.4% identity between Yop1p and murine TB2. It is notable that the overall structure of the mammalian and yeast protein is conserved. Both proteins contain extensive hydrophobic domains with the N terminus predicted to be exposed to the cytoplasmic face of the membrane. No other Yop1p homologs could be identified in *S. cerevisiae*.

Yop1p Is an Integral Membrane Protein—Sequence information predicts Yop1p to be a 20-kDa protein with two membrane-spanning segments that is likely oriented with its N terminus toward the cytoplasm (Fig. 1). We examined whether Yop1p has the expected properties of an integral membrane protein. First, Yop1p fractionated exclusively in the pellet of a total

A

B

C

D

Fig. 3. *A*, biochemical analysis of Yop1p and Yip1p interaction: GST-Yop1p interacts specifically with Myc_9-Yip1p. Lysates were prepared from cells expressing either GST-Yop1p and Myc_9-Yip1p (RCY425) or GST and Myc_9-Yip1p (RCY428). Detergent-solubilized lysates were incubated with GST beads for 30 min at 4 °C as described under "Experimental Procedures." After four washes, the bead-bound material was subjected to SDS-PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with both monoclonal 9E10 (1:500) to detect Myc_9-Yip1p and polyclonal anti-GST (1:800) to detect GST-Yop1p. Relevant protein marker sizes are indicated. Myc_9-Yip1p was detected on RCY425 but not on RCY428 after GST pull-downs. *B*, GST-Yop1p and Myc9-Yip1p interaction is specific to Yop1p and Yip1p. GST pull-down experiments as in *A* were performed on yeast expressing either GST-Yop1p and Myc_9-Yip1p (RCY425) or GST-Yop1p and Dss4p-Myc_3 (RCY429). The membrane was first probed with α-Myc to detect expression of Myc-tagged proteins and was subsequently probed with α-GST to confirm the presence of GST-Yop1p. Myc_9-Yip1p was detected after GST pull-down, but Dss4p-Myc_3 was not, indicating that the interaction is specific to Yip1p and Yop1p. *C*, GST-Yop1p specifically associates with Yip1p expressed at wild-type levels. Lysates were prepared from yeast expressing GST-Yop1p and either GFP-Yip1p (RCY509) or GFP-Ypt7p (RCY508) at single copy. Western blot analysis with affinity-purified anti-GFP antibody showed that GFP-Yip1p specifically associated with GST-Yop1p; however, a control protein (GFP-Ypt7p) expressed at similar levels did not associate with GST-Yop1p. Western blot of lysates demonstrates that the fusion proteins were expressed at equivalent levels in both strains. *D*, the complex of Yop1p and Yip1p interaction is formed *in vivo*. GST-Yop1p and Myc_9-Yip1p were either coexpressed in the same cell (RCY425) or expressed in different strains that were mixed after lysis (RCY429, RCY462). The GST-Yop1p and Myc_9-Yip1p interaction is only observed when the two constructs are expressed in the same cell, indicating that the interaction occurs prelysis or *in vivo*.
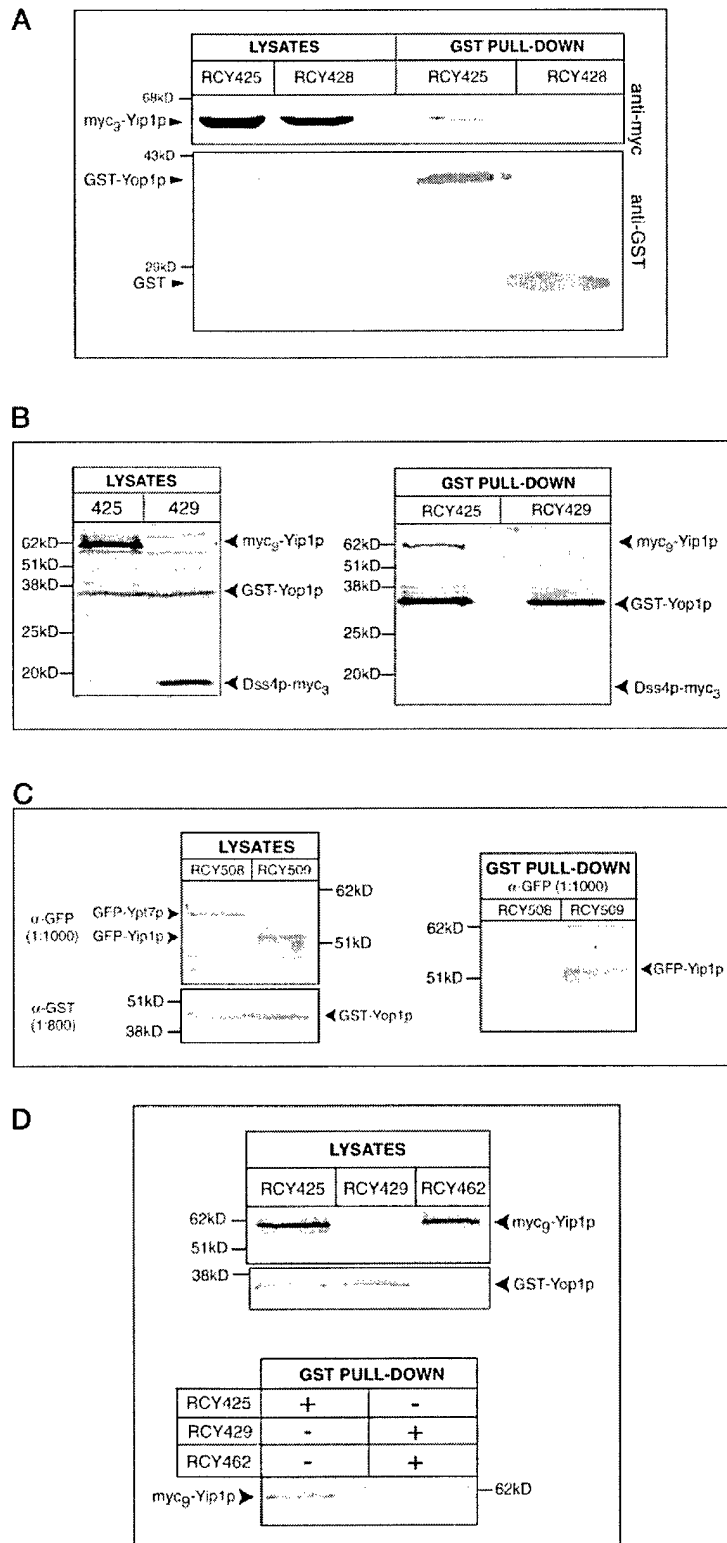
postnuclear supernatant centrifuged at 100,000 × *g*, indicating that it is either membrane-associated or present in a large pelletable aggregate (Fig. 2A). Second, we tested whether Yop1p was a peripheral membrane protein and could be re-

moved by washing membranes in high salt-containing buffers. Yop1p could not be extracted from membranes by incubation in buffer containing 1 M NaCl; however, Yop1p was quantifiably extracted in Triton X-100 detergent-containing buffers

A

B

C

D
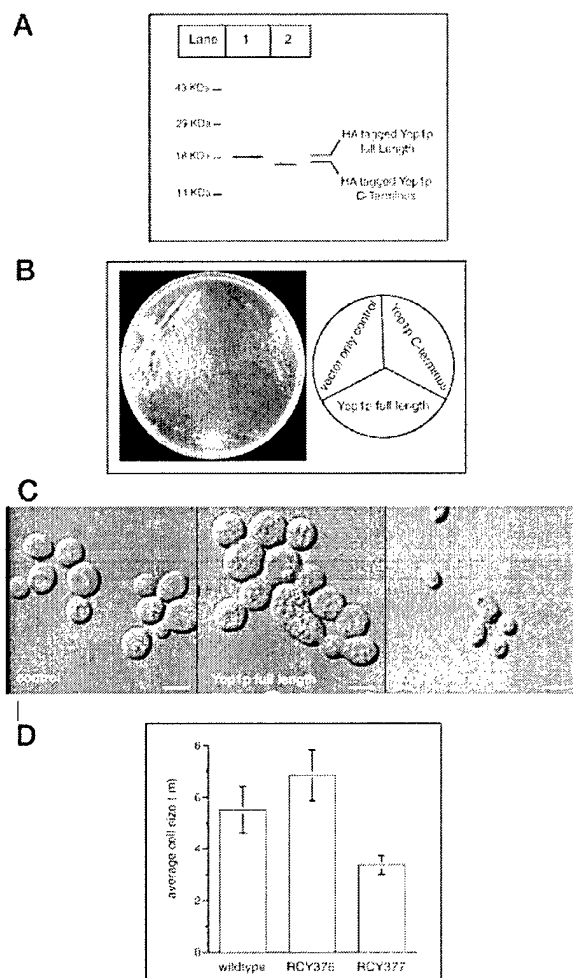
FIG. 4. **Overexpression of both Yop1p full-length and Yop1p C-terminal constructs results in a dominant negative phenotype.** *A* shows the ability of cells bearing constructs as indicated for galactose-dependent expression to grow when expression of the construct is induced by growth on galactose-containing medium after 3.5 days of growth at 30 °C. *B* shows a Western blot of lysates derived from cells shifted to galactose-containing medium for 10 h probed for the presence of the HA-tagged construct. *Lane 1* shows a lysate generated from RCY376 (Yop1p full-length construct), and *lane 2* shows a lysate generated from RCY377 (Yop1p C-terminal construct). *C* shows differential interference contrast (*DIC*) images of cells expressing the constructs as indicated and allowed to grow in galactose-containing medium for 2 days. All images are shown at the same magnification. *Bar*, 2.5 μm. *D* represents the quantification of cell size illustrated in *C*; for each condition, the width of randomly chosen cells was measured at the widest point, and the average size is shown together with the S.D.

(Fig. 2*B*). Third, we performed Triton X-114 phase extraction experiments to determine whether Yop1p has the physiochemical properties of an integral hydrophobic membrane protein. In this technique, total cellular proteins are first detergent solubilized at 0 °C. The mixture is then warmed to 30 °C, exploiting the cloud point of Triton X-114 to create two phases that can be separated by gentle centrifugation: a detergent-rich phase containing membrane proteins and an aqueous phase containing hydrophilic proteins. Yop1p partitioned exclusively into the detergent-rich phase (Fig. 2*C*), indicating that it contains hydrophobic domains that anchor it in the lipid bilayer. As a control, fractions were also probed for a known integral membrane protein, Snc1/2p (30), which partitioned into the detergent phase as expected. Taken to-

gether, these data show that Yop1p is an integral membrane protein.

Physical Association of Yip1p and Yop1p—To confirm the two-hybrid data, we performed biochemical studies of the Yip1p/Yop1p interaction. For this purpose, we created the strain RCY425, which expresses GST-Yop1p fusion protein under the control of the regulatable $GAL_{1/10}$ promoter and contains a multicopy plasmid expressing Myc_9-Yip1p. We also created an isogenic control strain, RCY428, which expresses GST alone together with Myc_9-Yip1p. Tween 20 detergent-solubilized total lysates were produced from mid-log phase cells grown in galactose and GST fusion proteins were isolated on gluthathione-agarose beads followed by SDS-PAGE and Western blotting to detect any associated Myc-tagged proteins. The results of this experiment are shown in Fig. 3*A*. Myc_9-Yip1p was detected in the GST-Yop1p pull-down but was not detected in the pull-down of GST alone, showing that Myc_9-Yip1p exists in physical association with Yop1p. To rule out any possibility of GST-Yop1p interacting with the Myc epitopes of Myc_9-Yip1p, we repeated the experiment with RCY429, which expresses GST-Yop1p together with $Dss4p$-Myc_3. In this experiment, the Western blot was first probed with anti-Myc antibody and then reprobed with anti-GST antibody. The results are shown in Fig. 3*B*. $Dss4p$-Myc_3, Myc_9-Yip1p, and GST-Yop1p are expressed at equivalent levels in the detergent-solubilized lysates. The GST pull-downs reveal that Myc_9-Yip1p associated with GST-Yop1p but $Dss4p$-Myc_3 did not associate and could not be detected in the pull-down, demonstrating that the biochemical association of Yip1p and Yop1p is specific.

We also repeated the experiment with Yip1p expressed at wild-type levels on a single-copy centromeric plasmid under the control of its own promoter and terminator. For these experiments, Yip1p was tagged with GFP, and lysates were produced with Triton X-100 detergent solubilization. Western blot analysis of the glutathione resin pull-downs (Fig. 3*C*) showed that GFP-Yip1p (RCY509) was specifically isolated with GST-Yop1p, while a control protein, GFP-Ypt7p (RCY508), was not. Western blots of the detergent-solubilized lysates confirmed that GST-Yop1p and the GFP fusion proteins were expressed at equivalent levels in both cases.

To further investigate the relationship between Yip1p and Yop1p, we asked whether the interaction in our pull-down experiments occurred *in vivo* prior to cell lysis or postlysis *in vitro*. For these experiments, we performed the glutathione resin pull-downs on lysates derived from cells coexpressing GST-Yop1p and Myc_9-Yip1p (RCY425) or by combining lysates from individual strains RCY429 (containing GST-Yop1p and $Dss4p$-Myc_3) or RCY462, which contains Myc_9-Yip1p only. These results can be seen in Fig. 3*D*. We were only able to detect the interaction of Yip1p and Yop1p from cells expressing both proteins simultaneously. These results indicate that Yip1p and Yop1p interact *in vivo*, in a complex that is formed prior to cell lysis.

Overexpression of YOP1 Is Dominant Negative and Can Be Suppressed by Co-overexpression of YIP1—We deleted the entire *YOP1* ORF in a diploid cell that was sporulated and dissected into tetrads to study the phenotype of the haploid-disrupted strain. The *YOP1Δ* haploids were viable, indicating that *YOP1* is dispensable for vegetative growth. Furthermore, a strain carrying the null allele has no apparent growth defect under several conditions commonly used to detect phenotypes in *S. cerevisiae* (31): high temperature (37 °C), low temperature (15 °C), 2 mM caffeine, 2% formamide, high salt (1 M NaCl), and glycerol as carbon source (data not shown). We next examined the phenotype of Yop1p overexpression. For this experiment, we expressed both full-length Yop1p (Yop1p full-length,

FIG. 5. **Multicopy *YIP1* can rescue the lethality associated with overexpression of full-length Yop1p but not of the Yop1p C-terminal construct that lacks the Yip1p-interacting domain.** RCY376 expresses full-length Yop1p, and RCY377 expresses the Yop1p C terminus in a galactose-dependent manner. The control strain was transformed with the GAL$_{1/10}$ HA-tagged vector only (no insert). Growth of these strains is shown on both glucose and galactose carbon sources with either multicopy *YIP1* or a control multicopy plasmid as indicated.
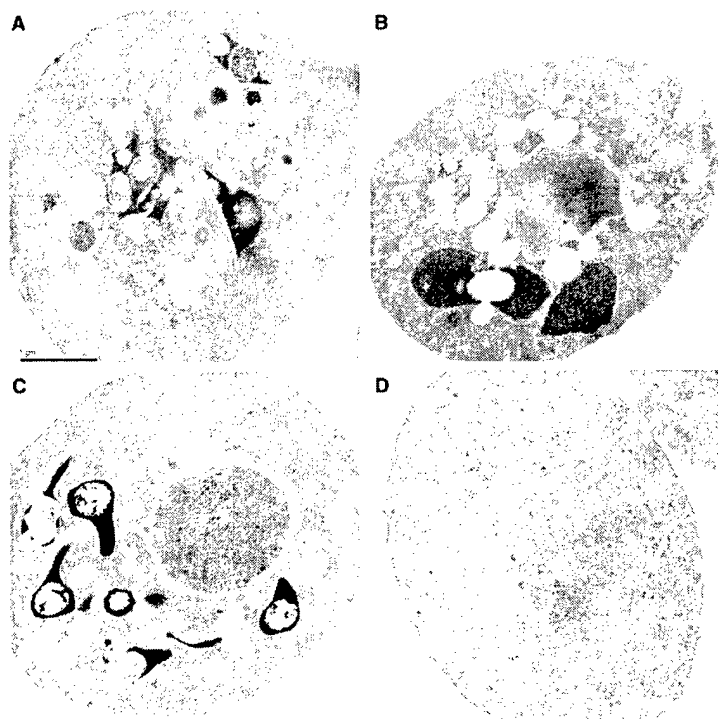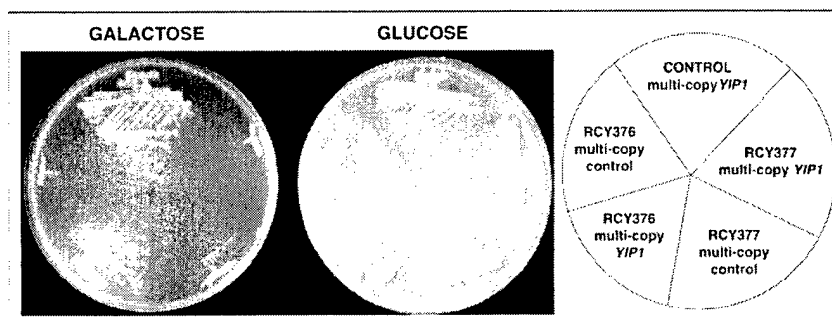

GALACTOSE   GLUCOSE

CONTROL multi-copy *YIP1*

RCY376 multi-copy control

RCY377 multi-copy *YIP1*

RCY376 multi-copy *YIP1*

RCY377 multi-copy control

A

B

C

D

FIG. 6. *A–D*, thin section electron microscopy of dominant negative *YOP1*. RCY376 cells expressing full-length dominant negative Yop1p (*A–C*) and NY605 cells expressing wild-type levels of Yop1p (*D*) were examined by thin section electron microscopy. Representative examples of each strain are shown. *Bar* in *A*, 1 μm. All *panels* are shown at the same magnification.

RCY376) and Yop1p that lacks 17 amino acids at the N terminus identified as the Yip1p-interacting region (Yop1p C terminus, RCY377). Both constructs were expressed in yeast as HA-tagged proteins under the control of the GAL$_{1/10}$ promoter. Immunoblot analysis of the lysates verified that both proteins were expressed in equivalent amounts upon shift of the growth medium to galactose (Fig. 4*A*). Both of these constructs were dominant negative for growth upon overexpression (Fig. 4*B*) but resulted in different morphologies. Overexpression of full-length Yop1p resulted in huge swollen cells of aberrant shape, while overexpression of the Yop1p C-terminal construct gave rise to much smaller cells, similar in shape but considerably smaller than cells harboring a control construct (Fig. 4*C*). To quantitate the observed effect, measurement of cell size was performed (Fig. 4*D*). Wild-type cells have an average width of 5.52 ± 0.90 μm; cells expressing full-length Yop1p have an average width of 6.85 ± 0.97 μm; and cells expressing Yop1p C-terminal construct have an average width of 3.37 ± 0.367 μm.

The dominant negative phenotype of full-length Yop1p overexpression was suppressed by co-overexpression of Yip1p from a multicopy plasmid (Fig. 5). However, multicopy *YIP1* had no effect on the dominant negative phenotype of the Yop1p C-terminal construct, which lacked the Yip1p interaction region.

These data further demonstrate that the interaction of Yip1p and Yop1p is a *bona fide* physiological interaction and support the identification of the Yop1p N terminus as the site of Yip1p interaction.

Dominant Negative YOP1 Results in Alteration of Membrane Structures and a Block in Membrane Traffic—To investigate morphological alterations in dominant negative *YOP1* cells in detail, we performed electron microscopy. Cells containing the full-length dominant negative *YOP1* construct and isogenic wild-type cells were grown in galactose before being fixed with and processed for electron microscopy. These results are shown in Fig. 6. Expression of the full-length *YOP1* dominant negative construct resulted in the disappearance of large vacuoles normally seen in wild-type cells and the appearance of smaller and aberrantly shaped compartments filled with darkly stained material (Fig. 6, *A–C*). These cells also contained numerous discontinuous ring-shaped structures; some membrane structures resembled the cup-shaped "Berkeley bodies" known to represent abnormal Golgi structures, while others had pleiomorphic, clublike shapes. In some cells, an accumulation of ER membranes, as judged by their connection to the nuclear envelope, was also observed. Such aberrant membrane structures are not observed in wild-type cells (Fig. 6*D*) and represent

A

B

FIG. 7. *A*, CPY immunoblot analysis of cells expressing dominant negative *YOP1* constructs. Shown is immunoblot analysis of total cell lysates for the relative level CPY processing in cells expressing dominant negative constructs containing full-length *YOP1* (RCY376), C-terminal *YOP1* (RCY377), and an isogenic control strain (RCY458). At the time points indicated after the switch from sucrose- to galactose-containing medium, samples were taken and processed for total cell lysates. The *arrows* indicate the relative migration of the p1 (core-glycosylated ER), p2 (Golgi-modified), and m (mature vacuolar) forms of CPY. *sec18* cells are shown at room temperature (permissive temperature) and after shift to 37 °C (restrictive temperature) for 1 h as a control for the migration of the various CPY forms and to provide a positive reference for the accumulation of p1 CPY, marked with *asterisk*. *B*, cell growth of dominant negative *YOP1*. Growth of cells expressing full-length *YOP1* dominant negative construct (RCY376) relative to isogenic wild-type strain (RCY458). Cells were grown to log phase in sucrose-containing selective medium before being switched to galactose-containing medium to induce expression of construct. At various times, as indicated, a turbidity measurement was made as a record of cell growth. Cell concentration was maintained in log phase for the duration of the experiment.
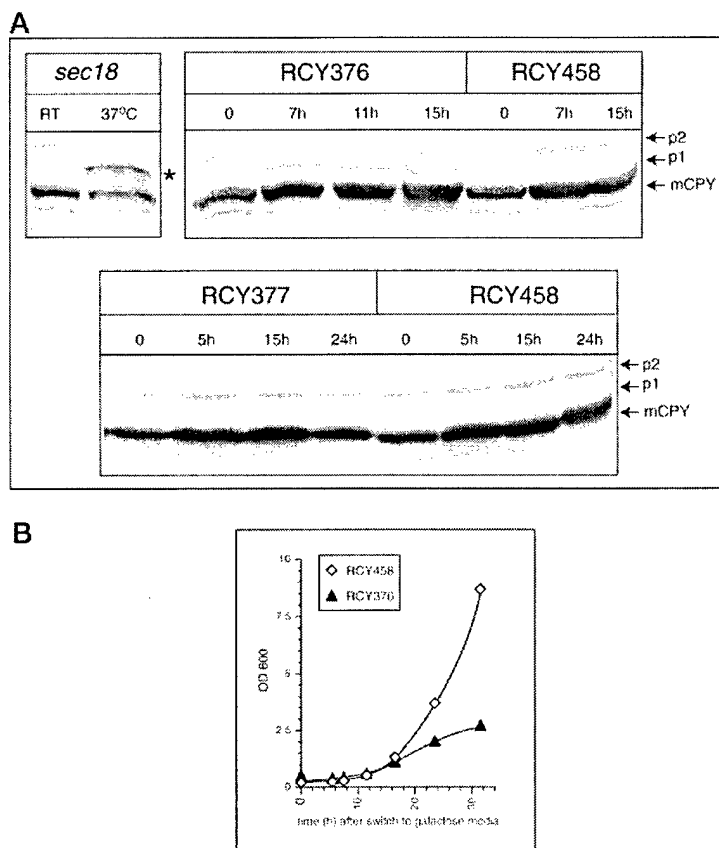
a gross distortion of the normal pathways of membrane traffic in the *YOP1* dominant negative cells.

To investigate the effect of *YOP1* overexpression on membrane traffic, we monitored the steady state level of newly synthesized precursors of the vacuolar protease CPY, the product of the *PRC1* gene. CPY is a soluble vacuole protein that undergoes processing from a core-glycosylated ER form (p1, 67 kDa) to a modified Golgi form (p2, 69 kDa) before being proteolytically cleaved in the vacuole to mature CPY (61 kDa). Using an anti-CPY antibody, we analyzed total cell lysates for the relative levels of the precursor and mature CPY forms under wild-type and YOP1 dominant negative conditions. As a control, we used *sec18* cells shifted to the restrictive temperature at which all stages of membrane traffic are blocked, resulting in the accumulation of the core-glycosylated p1 form of CPY (shown by an *asterisk*). The results are shown in Fig. 7A. Cells overexpressing full-length Yop1p show an accumulation of p1 CPY relative to isogenic wild-type cells (RCY458), which is indicative of a block early in exocytosis at the level of the ER. The observed accumulation is the specific result of a block in membrane traffic and does not reflect a generalized disruption of cellular function as the block can be observed within the first 7 h of galactose induction, cell growth rates are not affected until ~16.5 h after galactose induction (Fig. 7B). Dominant negative cells overexpressing Yop1p C terminus (RCY377) do not show the same effect, and no accumulation of CPY is observed.

Localization of YOP1—We examined the localization of Yop1p by subcellular fractionation and immunofluorescence. For this purpose, we constructed the strain RCY460, which contains wild-type levels of HA-tagged Yop1p as the sole cellular source of Yop1p. Separation of postnuclear supernatants

into P10 (after 10,000 × *g* centrifugation) and P100 and S100 (after 100,000 × *g* centrifugation) followed by immunoblotting with monoclonal anti-HA antibody (12CA5) is shown in Fig. 8A. HA-Yop1p fractionates with both light and heavy membranes (P10, P100) but not with cytosol (S100).

By immunofluorescence microscopy, HA-Yop1p appears as a punctate pattern that appears to be at, or near, the periphery of the cell, roughly proportionally distributed between the mother and bud with a greater concentration in the more actively growing region of the cell (Fig. 8B). To identify the cellular location of Yop1p, we performed double label immunofluorescence with Sec4p and with GFP-Ypt6p. HA-Yop1p does not localize to the bud tip or at the neck during cytokinesis and can be clearly distinguished from Sec4p immunofluorescence, which is solely concentrated at the leading edge of the cell (Fig. 9A). The Yop1p signal partially overlapped with the Ypt6p fluorescence, especially toward the leading edge of the cell, indicating the presence of Yop1p on Golgi membranes (Fig. 9B). The HA-Yop1p pattern of expression is identical whether or not the construct is integrated into the genome or maintained as a centromeric plasmid; the expression pattern is also identical in diploids and haploids and on cells grown in glucose, galactose, or glycerol carbon sources (data not shown).

To further examine the subcellular localization of Yop1p and its interactions with Yip1p, we performed confocal microscopy. Cells coexpressing HA-Yop1p and GFP-Yip1p are shown in Fig. 9C. Substantial overlap of the Yop1p and Yip1p signal was observed toward the growing edge of the cell, confirming our biochemical data indicating that a physical interaction between Yop1p and Yip1p occurs *in vivo*.

Interaction of Yop1p with Rab Proteins—Since Yip1p is required for secretory pathway function, presumably through its

A

14kD—

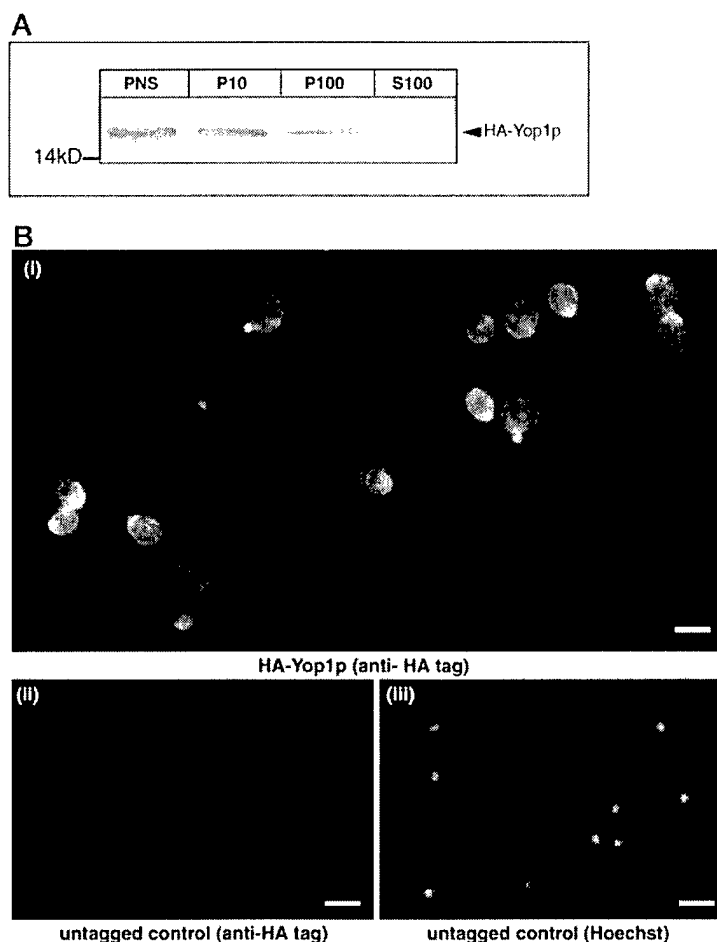| PNS	P10	P100	S100

◀ HA-Yop1p

FIG. 8. *A*, intracellular localization of Yop1p. RCY469 cells expressing wild type levels of a single HA epitope-tagged Yop1p as the only source of Yop1p were grown to logarithmic phase, disrupted with glass beads, and subjected to centrifugation at 500 × *g* to remove unbroken cells and cell debris. The PNS was fractionated by differential centrifugation at 10,000 × *g* to give pellet fraction P10 and 100,000 × *g* to yield pellet fraction P100 and supernatant fraction S100. Aliquots of fractions were subjected to SDS-PAGE and Western blot analysis with anti-HA monoclonal antibody. *B*, immunofluorescence localization of Yop1p. RCY469 cells expressing wild type levels of HA-tagged Yop1p as the only source of Yop1p (*A*) and an isogenic control strain, RCY407, expressing the untagged protein (*B* and *C*) were examined by immunofluorescence microscopy. HA-tagged Yop1p was localized with the anti-HA tag antibody Y11 (*A* and *B*). Nuclei were localized in the untagged control by Hoechst 33258 staining (*C*). All *panels* are shown at the same magnification. *Bar*, 5 µm.

B

HA-Yop1p (anti- HA tag)

untagged control (anti-HA tag) untagged control (Hoechst)

effects on Rab proteins, we sought to investigate any possible interaction of Yop1p with Rab proteins. There are 11 Rab protein family members in *S. cerevisiae*; however, some of these represent closely related isoforms (*e.g.* Vps21p, Ypt52p, and Ypt53p). To gain as complete an insight as possible, we made GST fusions with several Rab proteins encompassing representatives from each subset. These proteins were expressed in cells behind the galactose promoter and were tested for interaction by coprecipitation with Yop1p, which was tagged with a single HA epitope and also expressed behind the galactose promoter. The results of this analysis are shown in Fig. 10*A*. HA-Yop1p did not coprecipitate with GST alone but was able to precipitate with GST fused to the Rab proteins Ypt52p, Sec4p, Ypt6p, and Ypt7p. The observed interaction was stable in buffers containing 0.5% Tween 20; however, it could not be observed in 0.5% Triton X-100 containing buffers (data not shown). No interaction was observed with a Ypt1p construct lacking its C-terminal cysteines, which are the sites of prenylation. The interactions between Yop1p and Rab proteins are unlikely to be real *in vivo* interactions; otherwise, the steady-state localization of Yop1p would probably be more universally distributed among subcellular membranes. It is more likely that the observed interactions reflect a generalized biochemical ability of Yop1p to interact with a common determinant of fully post-translationally modified Rab proteins, an interaction that can be revealed by overexpressing both proteins and performing coprecipitation assays as shown in Fig. 10*A*. To reveal which Rab protein may be important for Yop1p action *in vivo*, we performed suppression analysis of the *YOP1* dominant negative

constructs with multicopy plasmids encoding all 11 Rab proteins of *S. cerevisiae*. The full-length *YOP1* dominant negative construct, while able to be suppressed by multicopy *YIP1* (Fig. 5), could not be suppressed by overexpression of any of the genes encoding the yeast Rab proteins (data not shown). However, multicopy *YPT6* was able to suppress the dominant negative phenotype of the *YOP1* C-terminal construct. The suppression of the *YOP1* C-terminal construct (RCY377) by 2µ *YPT6* together with *YPT7* and *DSS4* as a comparison is shown in Fig. 10*B*. *YPT6* was the only Rab gene capable of causing *in vivo* suppression of RCY377; no other Rab gene tested (*SEC4*, *YPT1*, *YPT31*, *YPT32*, *VPS21*, *YPT52*, *YPT53*, *YPT10*, *YPT11*, and *YPT7*; data not shown except for *YPT7*, Fig. 10*B*), was able to restore growth.

DISCUSSION

We have isolated *YOP1* as a novel *YIP1*-interacting clone in a yeast two-hybrid screen of a yeast genomic library. Yop1p and Yip1p are both integral membrane proteins. The interaction of a membrane protein in the two-hybrid system is perhaps surprising and worthy of comment. Yip1p is not alone in this regard; other membrane proteins have also been shown to functionally interact in such a system (29). Although some membrane proteins clearly cannot maintain their native structure and functional interactions in the two-hybrid system, there are at least two factors that might indicate whether or not the two-hybrid system will be useful for any given protein. (i) The GAL4 system contains a strong nuclear localization signal and so may dominate over other localization signals

Identification of Yop1p

A

Hoechst   Sec4p   Yop1p   merge

B

Hoechst   Ypt6p   Yop1p   merge

C

Yip1p   Yop1p   merge

Fig. 9. *A–B*, double label immunofluorescence microscopy of Yop1p with Sec4p and Ypt6p. RCY469 cells expressing wild-type levels of HA-tagged Yop1p as the only source of Yop1p together with wild-type levels of either GFP-tagged Sec4p or Ypt6p were examined by double label immunofluorescence microscopy. Cells were labeled with the anti-HA tag antibody Y11 to visualize HA-Yop1p (*A* and *B* (*i*)) and with anti-Sec4p (*A* (*ii*)) or anti-GFP (*B* (*ii*)). Nuclei were counterstained with the DNA stain Hoechst 33258 (*A* and *B* (*iii*)). A merge of all three channels is shown in *A* and *B* (*iv*). Note that under the processing conditions for immunofluorescence, there was no interference from the intrinsic GFP fluorescence. *C*, double label confocal immunofluorescence microscopy of Yop1p with Yip1p. RCY496 cells expressing wild-type levels of HA-tagged Yop1p as the only source of Yop1p together with wild-type levels of GFP-tagged Yip1p were examined by double label confocal microscopy. Cells were labeled with anti-HA tag antibody (*i*) and with anti-GFP antibody (*ii*). A merge of both channels is shown in *iii*.
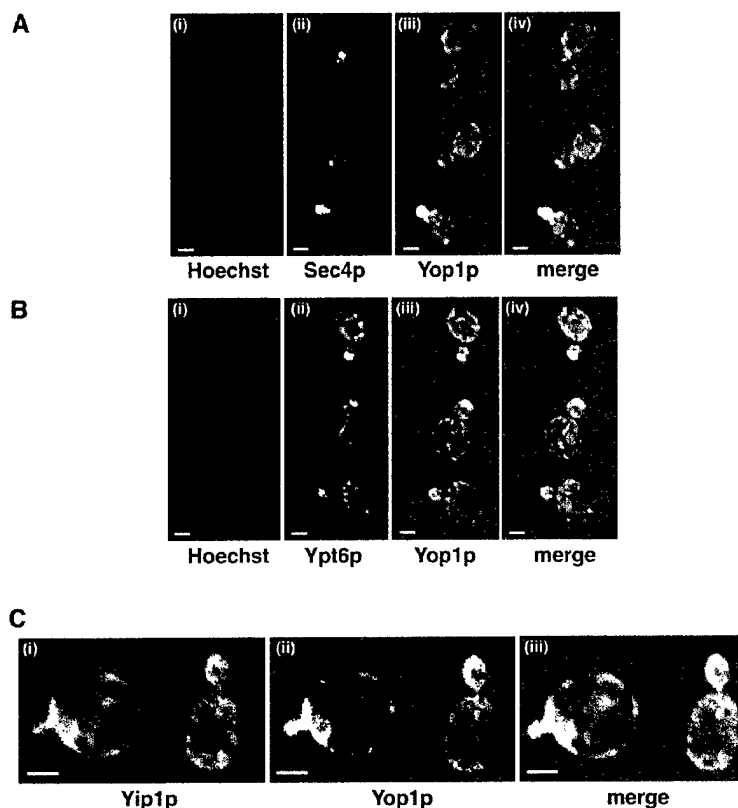
present in the two-hybrid construct and be a better system for this purpose than a system that relies on passive diffusion to enter the nucleus (32). (ii) *S. cerevisiae* is probably more capable of correctly folding endogenous yeast proteins rather than proteins from other organisms. In addition, membrane channels have been observed in the nucleus (33), and some viruses acquire membranes in the nucleus (34), indicating that the ultrastructure of the nucleus may be more complex than originally thought.

We have identified two functional domains of Yop1p that act in a dominant manner to inhibit cell growth upon overexpression. The first domain consists of the cytosolic N terminus of Yop1p that corresponds to the first exon of the *YOP1* gene. The second domain comprises the C terminus of the molecule that is mainly hydrophobic and corresponds to the second genomic exon. Overexpression of full-length Yop1p leads to inhibition of cell growth and a phenotype of enlarged cells that accumulate internal membrane structures. This phenotype can be suppressed by co-overexpression of Yip1p. Presumably, Yip1p is able to suppress the toxic effect of Yop1p by sequestration. These data would suggest that *YOP1* overexpression inhibits cell growth by inhibiting the function of Yip1p, since Yip1p is an essential gene required for secretion. Consistent with this interpretation is the phenotype of *YOP1* full-length overexpression, which results in the accumulation of membrane structures and an accumulation of the ER core-glycosylated form of CPY, indicating a block in ER to Golgi traffic. Recently, a Yip1p homolog, Yif1p, has been identified (35) that appears to act similarly to Yip1p in blocking ER to Golgi transport. Although nothing is known about the precise function of Yip1p, the identification of Yif1p and Yop1p as Yip1p binding partners suggests that Yip1p may be involved in several different Rab-mediated events through a combinatorial assortment with different binding partners.

The phenotype of the *YOP1* C-terminal construct is distinct

from that of the full-length construct. Yip1p co-overexpression cannot suppress the dominant negative effect of Yop1p C terminus overexpression. The Yop1p-C-terminal construct lacks the domain that is both necessary and sufficient for Yip1p interaction by two-hybrid analysis. The mechanism by which this construct inhibits growth cannot be directly via an inhibition of Yip1p function. One clue may be provided by the fact that *YPT6* can suppress the dominant negative *YOP1* C-terminal construct but not that of the full-length construct, indicating that the action of Yop1p is intimately connected to Rab function. This finding further underscores our results, demonstrating that Yop1p can be specifically coprecipitated with Rab proteins in cellular lysates. Since Yop1p shows a restricted sub-cellular localization, we hypothesize that the biochemical interaction of Yop1p with Rab proteins is limited *in vivo*, possibly only to *YPT6*, which our suppression analysis demonstrates to interact genetically with *YOP1*. Consistent with this interpretation are our data demonstrating that the steady-state immunofluorescent localization of Yop1p and Ypt6p shows overlap *in vivo*.

The two domains of Yop1p may act antagonistically, or perhaps the exposed Yop1p N terminus may constitute a signaling domain that acts in a dose-dependent manner to negatively regulate membrane traffic. There is a growing appreciation that many proteins involved in the regulation of intracellular membrane traffic may act as signal transducers that coordinate membrane traffic with other cellular events (36–38). Different branches of the Ras superfamily are ideally placed to coordinate such cross-talk, and our data indicate that *YOP1* and possibly its human homolog *TB2* may also play a role in the regulation of cell growth through its facilitation of membrane traffic. Our genetic data suggest that *YOP1* is a recessive gene that negatively regulates cell growth. Deletion of *YOP1* has no apparent effect on cell viability, and full-length and C-terminal *YOP1* constructs

FIG. 10. *A*, coprecipitation of Yop1p with Rab proteins. Lysates were prepared from cells expressing either GST alone or various GST-Rab constructs as indicated, together with HA-tagged-Yop1p. Detergent-solubilized lysates containing 0.5% Tween 20 were incubated with GST beads for 30 min at 4 °C as described under "Experimental Procedures." After four washes, the bead-bound material was subject to SDS-PAGE electrophoresis and analyzed by Western blotting. Membranes were probed with both polyclonal anti-GST (1:800) to detect the bead-bound GST fusion proteins and monoclonal 12CA5 to detect any associated HA-Yop1p. Relevant protein marker sizes are indicated. All constructs were under the control of the $GAL_{1/10}$ promoter and were expressed by inducing with galactose for ~8 h. HA-Yop1p was detected associated with Ypt52p, Ypt6p, Sec4p, and Ypt7p GST fusion proteins but not on GST alone or Ypt1p lacking its C-terminal cysteines. *B*, growth of dominant negative YOP1 C-terminal construct (RCY377) with various plasmids. RCY377 expresses the Yop1p C terminus in a galactose-dependent manner. The control strain was transformed with the $GAL_{1/10}$ HA-tagged vector only (no insert). Growth of these strains is shown on both glucose and galactose carbon sources with either multicopy *YPT6*, *YPT7*, or a control multicopy plasmid as indicated.
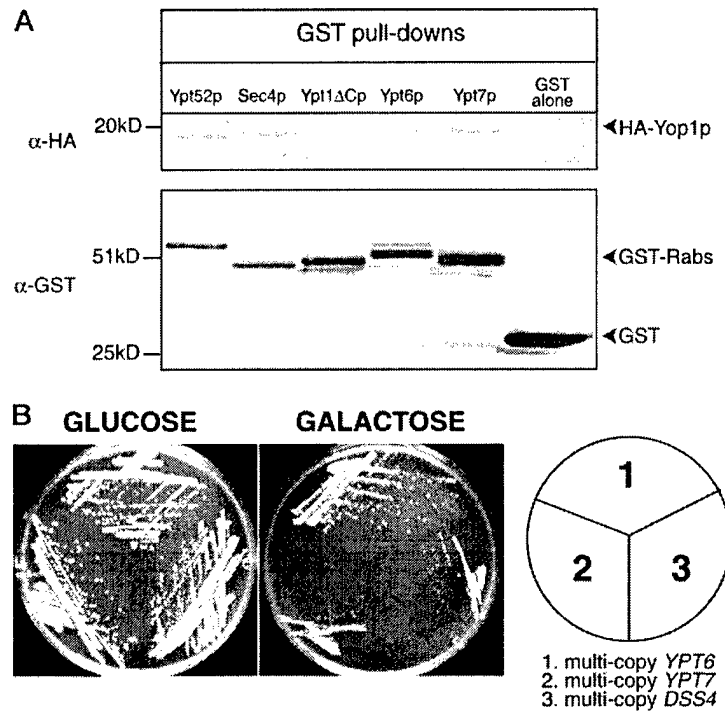
possess a dose-dependent growth inhibitory effect.

Sequence comparison revealed that Yop1p is homologous to the human *TB2* gene with sequence similarities throughout the protein. The amino acid sequence conservation of Yop1p across species clearly points to its functional importance, and an interesting finding is that *TB2* is a human familial adenomatous polyposis locus (39) gene (40), adjacent to the tumor suppressor genes *MCC* and *APC* (41). *TB2* encodes a 197 amino acid polypeptide (1). The deduced amino acid sequence predicts that Yop1p contains at least two extensive membrane-spanning segments. The human *TB2* gene also contains a similar size and type of membrane-spanning segments and would be predicted to have the same topology. This similarity raises the possibility that Yop1p and TB2 may share a common function in mediating vesicular transport. It is now clear that the machinery and mechanisms of membrane traffic share much in common between yeast and higher eukaryotes (42). For example, the complex observed between Sec9p, Sso1/2p, and Snc1/2p, which is required for exocytosis in yeast, is the structural and functional counterpart of the neuronal SNARE complex (30). Rabs are also extremely well conserved over evolution. In some cases, yeast and mammalian Rab proteins are functionally interchangeable. For example, Vps21p/Ypt51p, a homolog of mammalian Rab5, is also required at an early step in endocytic traffic (43). Remarkably, Ypt51p expression in animal cells not only localizes to Rab5-positive early endosomes but also stimulates endocytosis (44). This latter fact indicates that the machinery involved in mediating Rab protein function is probably conserved across diverse species. Our data indicate that Yop1p, probably in conjunction with Yip1p, acts to facilitate a Rab-mediated event in membrane traffic. It remains to be demonstrated whether TB2 has a role in membrane transport.

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